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Pitx1 determines the morphology of muscle, tendon, and bones of the hindlimb

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Abstract

The vertebrate forelimb and hindlimb are serially homologous structures; however, their distinctive morphologies suggest that different mechanisms are associated with each limb type to give rise to limb-type identity. Three genes have been implicated in this process; T-box transcription factors *Tbx5* and *Tbx4*, which are expressed in the forelimb and hindlimb, respectively, and a paired-type homeodomain transcription factor *Pitx1*, expressed in the hindlimb. To explore the roles of *Pitx1* and *Tbx4* in patterning the hindlimb, we have ectopically misexpressed these genes in the mouse forelimb using transgenic methods. We have developed a novel technique for visualising the structure and organisation of tissues in limbs in 3D using optical projection tomography (OPT). This approach provides unparalleled access to understanding the relationships between connective tissues during development of the limb. Misexpression of *Pitx1* in the forelimb results in the transformation and translocation of specific muscles, tendons, and bones of the forelimb so that they acquire a hindlimb-like morphology. *Pitx1* also upregulates hindlimb-specific factors in the forelimb, including *Hoxc10* and *Tbx4*. In contrast, misexpression of *Tbx4* in the forelimb does not result in a transformation of limb-type morphology. These results demonstrate that *Pitx1*, but not *Tbx4*, determines the morphological identity of hindlimb tissues.
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Introduction

During development, the forelimb and hindlimb buds are morphologically uniform, however, as limb development proceeds, unique structures form characteristic of each limb type. For example, in the forelimb, chondrocytes, myoblasts, and tendon cells are directed to form an elbow joint, whereas in the hindlimb, the equivalent cell populations give rise to a knee. The signaling mechanisms that ultimately produce the distinct limb-type morphologies remain unknown. Gaining an understanding of these processes will allow insights into how limb-type identity arises, and in a broader context, how identical cell populations and common signaling cascades are modulated to generate diversity of form.

Three transcription factors that are expressed in a limb-type-restricted manner have been implicated in limb identity. *Pitx1*, a paired-type homeodomain transcription factor, is expressed in a hindlimb-restricted manner (Logan and Tabin, 1999; Logan et al., 1998; Shang et al., 1997; Szeto et al., 1999). In mice lacking *Pitx1*, hindlimb outgrowth is slightly impaired, but evidence of a loss of some hindlimb features supports a role for this factor in determining hindlimb morphology (Lanctot et al., 1999; Marcil et al., 2003; Szeto et al., 1999). T-box transcription factors, *Tbx5* and *Tbx4*, are expressed in the forelimb and hindlimb, respectively, in several species (Chapman et al., 1996; Gibson-Brown et al., 1996; Isaac et al., 1998; Logan et al., 1998; Ruvinsky et al., 2000; Simon et al., 1997; Takabatake et al., 2000). Inactivation of these factors leads to a failure of limb formation, indicating that they are required for initiation of the limb (Agarwal et al., 2003; Ahn et al., 2002; Naiche and Papaioannou, 2003; Rallis et al., 2003).

In the chick, misexpression of *Pitx1* leads to a transformation of forelimb structures to reflect characteristics of hindlimbs, directly implicating this factor in specifying hindlimb identity (Logan and Tabin, 1999). Misexpression of *Tbx5* and *Tbx4* in

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the chick limb has also been reported to result in transformations of the hindlimb and forelimb, respectively (Rodriguez-Esteban et al., 1999; Takeuchi et al., 1999). However, recent work in the mouse has shown that *Tbx4* can replace *Tbx5* function in the forelimb but does not transform the forelimb to a hindlimb, suggesting *Tbx5* and *Tbx4* have a primary role in initiating limb outgrowth, but do not determine limb-type (Minguillon et al., 2005). Replacement of *Tbx5* with both *Tbx4* and *Pitx1*, results in a forelimb with hindlimb-like characteristics, further implicating *Pitx1* in determining hindlimb identity (Minguillon et al., 2005).

To understand the role of *Pitx1* in determining limb-type identity in the mouse, we have investigated its effects using a construct where the transgene is ectopically expressed in the forelimb and over-expressed in the hindlimb using the *Prx1* limb enhancer (Martin and Olson, 2000). As a comparison, we have also investigated the effects of ectopic misexpression of *Tbx4* in the forelimb.

To understand the role of these genes in determining the identity of individual muscles, tendons, and bones of the limb, we have developed a novel method for visualising the complex organisation of these structures in 3D. Using reporter lines, immunohistochemistry, and optical projection tomography (OPT) (Sharpe et al., 2002), we can resolve individual muscles, tendons, and bones and study their shape and position in the forming limb. We have used OPT to generate detailed 3D models and “virtual” sections of limbs that has enabled us to describe changes in attachment and position of muscles and tendons and the identity of skeletal elements resulting from misexpression of *Pitx1*. This is the first use of this approach for studying the forming limb. Our results demonstrate the enormous potential of this technique for future analysis of limb development and the development of other organ systems.

Misexpression of *Pitx1* results in transformation of some skeletal elements, muscles, and tendons of the forelimb to reflect hindlimb characteristics. This includes transformation of the elbow to a knee-like joint, carpal bones of the wrist to tarsal-like bones, and the transformation and translocation of forelimb muscles and tendons to resemble muscles in the hindlimb. In contrast, misexpression of *Tbx4* in the forelimb does not result in similar patterning changes. In *Pitx1* transgenics, hindlimb-restricted factors *Hoxc10* and *Tbx4* are ectopically expressed in the forelimb, whereas no similar changes in gene expression were observed following *Tbx4* misexpression in the forelimb.

Ectopic expression of *Pitx1* in the forelimb also results in downregulation of factors involved in anterior–posterior patterning of the limb, including *Shh* and *Ptc*, and a corresponding loss of posterior digits, whereas over-expression in the hindlimb results in a normal limb.

Materials and methods

Transgenic mice

Pitx1 and *Tbx4* were ectopically misexpressed under the *Prx1* limb enhancer (Martin and Olson, 2000). *Prx1–Pitx1* and *Prx1–Tbx4* transgenic lines were generated as described previously (Minguillon et al., 2005). Hemizygotes of both *Prx1–Pitx1* and *Prx1–Tbx4* lines are viable and fertile and were used for subsequent breeding and embryo harvests. Homozygote pups die at birth. Tendons were visualised with the *Scleraxis(Scx)-GFP* reporter line (R. Schweitzer, unpublished). Cartilage was visualised with the *Collagen2(Col2)-GFP* reporter W. Horton (Cho et al., 2001; Grant et al., 2000) (Shriners Hospital, Portland, Oregon).

Harvesting and genotyping embryos

Mouse embryos were staged according to Kaufman (1992). Noon on the day a vaginal plug was observed was taken as E0.5 day gestation. Embryos used for RNA *in situ* hybridisation were harvested at E10.5 and E12.5. Embryos for immunohistochemistry and OPT analysis were harvested at E14.5 and immediately ex-sanguinated by severing the umbilical cord in medium containing heparin (10 mg/ml, Sigma-Aldrich, U.K.) at 37°C for 1 h. Newborn pups used for skeletal preparations were culled at birth. All specimens were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) with 1% Tween (PBT) at 4°C. E14.5 embryos were fixed for 2 h, and E10.5 and E12.5 embryos and newborn pups were fixed overnight. Specimens were washed in PBT, and E10.5 and E12.5 embryos and pups were dehydrated in graded methanol and stored at 4°C. E14.5 embryos were stored in PBT at 4°C until use.

Prx1–Tbx4 and *Prx1–Pitx1* transgenic embryos were identified by PCR genotyping (Minguillon et al., 2005). To distinguish hemizygote from homozygote embryos at E10.5, real-time quantitative PCR was performed in multiplex mode using two sets of primers and probes for the *SV40* transgene (forward: 5′-GATTCCAACCTATGGAACTGA; reverse: 5′-GGCATTTCTTCTGAGCAAAAC; probe: 5′-VIC-TGGGAGCAGTGGTGGAATGCCTTTA-TAMRA), and as a control, the mouse cardiac actin promoter (α*CA*) (forward: 5′-CCCCCTGGCTGATCCTCTAC; reverse: 5′-TGGTCGCCTTAGCACCATCT; probe: 5′-FAM-CTCCAAGAATGGCCTCAGCGGTCC-TAMRA) (Tesson et al., 2002). The presence of *Scx-GFP* and *Col2-GFP* transgenes was identified by examination under fluorescent light.

Skeletal preparations

Cartilage and bone of newborn pups was stained using Alcian blue and alizarin red as described (Hogan et al., 1994).

Whole-mount double immunohistochemistry

Limbs were removed from E14.5 embryos and skin was removed using forceps under a light microscope. Samples were blocked in PBS containing 1% bovine serum albumin (BSA), 0.15% glycine, and 0.1% Triton for 2 h at room temperature prior to application of antibodies. Muscles were detected using a monoclonal antibody to fast skeletal muscle myosin (*My-32*, Sigma). Unconjugated *My32* antibody was directly conjugated with Texas red-labeled *Fab* fragments (Zenon™ One Texas Red-X Mouse IgG, Molecular Probes, Oregon, U.S.A.) at 1:800 dilution. GFP was detected using a primary rabbit polyclonal anti-GFP antibody (Molecular Probes), detected with a secondary donkey anti-rabbit antibody conjugated to FITC or Texas Red (Jackson ImmunoResearch Laboratories, Ltd., West Grove, PA, USA), both at 1:400 dilution. Diluted antibodies were applied to specimens in blocking agent (as above) overnight at 4°C. Samples were washed in PBS containing 0.1% Triton for 5 h following application of each antibody, and post-fixed for 30 min in 4% PFA at room temperature.

In situ hybridisation

Whole-mount in situ hybridisations were carried out as previously described (Riddle et al., 1993). All mouse probes have been previously described: Gli3 (Schimmang et al., 1992), Hoxb8 (Charite et al., 1994), Hoxc4, Hoxc5, (Burke et al., 1995), Hoxc10, Hoxc11 (Peterson et al., 1994), MyoD (Davis et al., 1987), Ptc1 (Goodrich et al., 1996), Scx (Schweitzer et al., 2001), Shh (Echelard et al., 1993), Tbx4, Tbx5 (Logan et al., 1998).

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